Activity of Chlorine Dioxide in a Solution of Ions and pH Against *Thielaviopsis basicola* and *Fusarium oxysporum*

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ABSTRACT

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Chlorine dioxide (ClO_2) is a disinfestant used to control pathogens in water. To determine if interactions between inorganic ions and pH levels effect ClO_2 activity in vitro, concentrations of ClO_2 (0, 1, 3, 5, 7, 9, 22, 24, 46, 58, and 70 mg/liter) were mixed for 10 min in solutions containing a nitrogen and hard water solution with equal concentrations of ammonium, nitrate, and synthetic hard water (0 and 100 mg/liter) and a divalent metal ion solution with equal concentrations of copper, iron, manganese, and zinc (0, 1, 3, and 5 mg/liter) at pH 5 and 8. Macro- and microconidia of *Fusarium oxysporum* f. sp. *narcissi* or conidia and aleuriospores of *Thielaviopsis basicola* were injected into each suspension for 30 s, captured on filter paper disks that were flushed with water, and plated on 50% potato dextrose agar. Spore germination was quantified after 1 day. ClO_2 activity had a similar effect on both fungal species and all types of propagules with interactions among the divalent metal ion solution, nitrogen and hard water solution, and pH treatments. A higher concentration of ClO_2 was required at pH 8 than at pH 5 to achieve a lethal dose resulting in 50% mortality of spores (LD_{50}). The addition of the divalent metal ion solution required an increase in ClO_2 concentration to maintain a LD_{50}. When combined with the nitrogen and hard water solution, the divalent metal ion solution placed a higher demand on ClO_2 at pH 5 and a lower demand on ClO_2 at pH 8, thus requiring an increase and decrease in a ClO_2 concentration, respectively, to achieve a LD_{50}. Chlorine dioxide doses resulting in 50% mortality ranged from 0.5 to 7.0 mg/liter for conidia of *F. oxysporum*, 0.5 to 11.9 mg/liter for conidia of *T. basicola*, and 15.0 to 45.5 mg/liter for aleuriospores of *T. basicola*.

Additional keywords: black root rot, *Chalara*, Fusarium basal rot

Chlorine dioxide (ClO_2) is a disinfestant approved for treatment of public and private drinking water and of poultry effluent at processing facilities, as well as for meat processing (fish, red meat, and poultry) facilities, postharvest handling of fruits and vegetables, and in some states, for postharvest treatment of potatoes in storage (22, EPA Reg. No. 5382-43-43553). Forms of chlorine other than ClO_2 (sodium and calcium hypochlorite and chlorine gas) are commonly used by ornamental plant producers. However, ClO_2 is chemically different from other chlorine disinfestants (1,7,8,16,17,21). Chlorine dioxide has an oxidative potential 2.5 times greater than hypochlorites and second only to ozone (1,8). Hypochlorites and chlorine gas hydrolyze rapidly in water to form hypochlorous acid, while ClO_2 does not hydrolyze appreciably in water but remains in solution as a gas. Chlorine dioxide has a

solubility five times that of chlorine gas and forms a monomeric structure that is more stable in water suspension than other chlorine products (17). Hypochlorites and chlorine gas react by oxidation and electrophilic substitution, while chlorine dioxide reacts only by oxidation, generally as an electron acceptor. As a result, hydrogen atoms present in activated organic structures are not substituted by chlorine dioxide. Therefore, chlorinated organic by-products are less commonly produced with ClO_2 treatments than when treating with other forms of chlorines, and low to negligible levels of chlorophenols, halo acetic acids, and trihalomethanes are formed. The latter is a primary reason for the increased use of ClO_2 for treating drinking water.

One disadvantage of ClO_2 over other chlorine products is the cost of a computer-monitored system to generate ClO_2 on-site. Chlorine dioxide can be generated using any of a number of methods, and delivery systems vary with application from treatment of drinking water to industrial wastes. Three delivery systems are currently commercially available for agricultural uses (22,24).

Chlorine dioxide exhibits biocidal activity against a range of organisms, including algae, animal planktons, bacteria, fungi,

and viruses (4,14,15,18,19,24). Researchers have demonstrated that high biocidal activity was obtained from ClO_2 with concentrations and duration of exposure that ranged from 1 to 9 mg/liter and 1 to 20 min, respectively. For example, a high reduction in viable propagules resulted when conidia or sporangiospores of *Botrytis cinerea*, *Penicillium expansum*, *Mucor piriformis*, and *Cryptosporiopsis perennans* were exposed to ClO_2 at 3 to 5 mg/liter for 1 min, and when *Phytophthora cinnamomi*, *Fusarium oxysporum*, *Colletotrichum capsici*, *Pythium ultimum*, and *Alternaria zinniae* were exposed to ClO_2 at 3 mg/liter for 8 min (18,24). These papers demonstrated that concentration of ClO_2 varies with time, with an equal mortality of propagules obtained at lower concentrations of ClO_2 by lengthening the duration of exposure. The upper rate of 9 mg ClO_2 per liter is higher than rates commonly used to treat drinking water or in fruit and vegetable dump tanks, where rates of 2 to 5 mg/liter are commonly used.

The effectiveness of disinfestants also depends on the properties of water (pH, water hardness, suspended solids, inorganic and organic molecules, turbidity, and temperature) and the treatment process, which affect the distribution of disinfestant in the body of water and the duration that molecules with biocidal activity are in contact with target organisms (1,7,8,10,17). All disinfestants, including ClO_2, react rapidly with various molecules, elements, particles, and organisms in suspension. These reactions create a demand (termed demand load) that lowers the concentration available to contact, be absorbed by, and inactivate micro-organisms. Chlorine dioxide is oxidized by Fe^{2+} and Mn^{2+} ions to precipitate ferric hydroxide and manganese oxide, respectively (7). Chlorine dioxide does not react with ammonia, bromides, nitrate, hard water, and zinc (5,7). Although ClO_2 is primarily used as an oxidizing agent, it is both oxidized and reduced under alkaline conditions, a process termed disproportionation, and the rate is rapidly accelerated at pH $\geq$ 10 (12,27).

Water hardness used to be measured by the capacity of water to precipitate soap (5). Current numerical values of water hardness are primarily the sum of carbonate and bicarbonate alkalinity, collectively termed "carbonate hardness" (5). Carbonate hardness is chiefly due to the presence of calcium and magnesium ions. Addi-

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tional hardness has been attributed to complex polyvalent cations associated with organic constituents, and is termed "noncarbonate hardness". Noncarbonate hardness can be difficult to define and is thought to be of minimal influence in water systems. In this research project, synthetic hard water will be strictly due to carbonate hardness. No references were found by the authors that noted an interaction between water hardness and ClO_2 activity. Water hardness of 400 mg/liter did not inhibit activity from 5 mg/liter available chorine derived from sodium hypochlorite (26).

A small survey of public leaflets distributed by city water authorities (Atlanta, GA; Bakersfield, CA; Bellingham, WA; Boston, MA; Columbus, OH; Everett, WA; Hattiesburg, MS; Mt. Vernon, WA; Olympia, WA; Puyallup, WA; Spokane, WA; Tacoma, WA; and Wenatchee, WA) about public drinking water showed that pH ranged from 5.6 to 10.1, water hardness ranged from 1 to 222 mg/liter carbonate hardness, nitrates ranged from 0.001 to 18.000 mg/liter, and minerals such as iron and manganese ranged form 0.0001 to 0.5000 mg/liter. These properties may vary to a greater degree in unregulated water sources, and elemental levels from nutrient leachates may be higher in some catchment ponds (7).

Chlorine dioxide can be used to disinfest irrigation water used in nursery and greenhouse ornamental plant production systems. Since cost of a chemical is a consideration in choosing a disinfestation system, and ClO_2 systems also require an initial investment of a computerized mixing and injection system, demand load responses that affect usage rates must be understood. Our objective was to determine the relative degree of interaction between inorganic ions and pH that affect the biocidal activity of ClO_2, measured as a function of concentration with a set exposure period (30 s) against several types of propagules from two fungi. Micro- and macroconidia of *Fusarium oxysporum* f. sp. *narcissi* (Cooke & Massee) and conidia of *Thielaviopsis basicola* (Berk. & Broome) Ferraris (syn. *Chalara elegans* Nag Raj & Kendrick) were selected to provide a diversity of hyaline spore types. The thick-walled, melanized aleuriospores of *T. basicola* were used as a propagule type that potentially had low sensitivity to ClO_2. The information generated from this research indicates whether a narrow or wide range in rate of ClO_2 is needed for efficacy against different fungal propagules as a result of the interactions of water properties (e.g., pH and water hardness) that vary with the source (e.g., ponds, wells, municipal water, and recirculating systems), in combination with the presence of nutrient leachates that collect in catchment ponds and ebb-and-flow systems.

MATERIALS AND METHODS

Inoculum. Chlamydospores of *F. oxysporum* f. sp. *narcissi* (isolate KMH-3 obtained from a 'Mt. Hood' daffodil bulb in 1993) were produced and stored in talc as described by Price (23). The talc–inoculum mixture was stored in plastic jugs at room temperature until inoculum was needed to start a culture. Cultures were grown on potato dextrose agar (PDA) for 10 to 14 days under cool fluorescent lights. Conidial suspensions were made by flooding plates with 10 ml of sterile, deionized water, scraping the surface with a sterile rubber policeman, and swirling and filtering the suspension through four layers of sterile cheesecloth. The plate was flooded a second time with 5 ml of water and the process repeated. Spores were enumerated using a hemacytometer and adjusted to obtain 1.0×10^6 conidia per ml in a 20-ml suspension. The amount of spore suspension added to 20 ml of a treatment solution averaged 0.28 ml. Spore suspensions were made daily as needed. Inoculum intended for use 2.5 h after preparation was subdivided into separate sterile beakers, covered with sterile aluminum foil, stored at 5°C, and returned to room temperature 15 min before use.

T. basicola (isolated from roots of *Fuchsia × hybrida*) was grown on V8 agar for 14 to 18 days to obtain predominately conidia, and on Difco PDA (Benton Dickson and Co., Sparkes, MD) with 2% yeast extract for 18 to 24 days to obtain aleuriospores. Spore suspensions that consisted predominately of conidia were prepared as described for *F. oxysporum* f. sp. *narcissi*. For suspensions that contained predominately aleuriospores, the agar was flushed with a gentle stream of sterile, deionized water using a wash bottle to remove most of the conidia, then inoculum preparation procedures were followed as described for *F. oxysporum* f. sp. *narcissi*.

pH, nitrogen, water hardness, and micronutrients. Certified buffer solutions (Fisher Scientific, Pittsburgh, PA) were used for pH 5 and 8. The ammonium, nitrate, copper, iron, manganese, and zinc solutions were made with ammonium sulfate $((NH_4)SO_4)$, potassium nitrate (KNO_3), copper sulfate $(CuSO_4 \cdot 7H_2O)$, manganese sulfate $(MnSO_4 \cdot H_2O)$, and zinc sulfate $(ZnSO_4 \cdot 7H_2O)$. Chelated iron was prepared by: dissolving iron sulfate (84 g $FeSO_4 \cdot 7H_2O$) in 1 liter of deionized water; dissolving ethylenediamine tetraacetic acid (84.6 g EDTA-disodium salt) in a second liter of deionized water; adding sodium hydroxide (10 g NaOH) to the EDTA solution; mixing the two solutions; adjusting total volume of the solution to 3 liters with deionized water; and holding it in the dark for 24 h with air gently bubbling through the solution. The final solution was a clear, tan-brown color. Precipitate formed 2 days after making the solution. Clarity of the solution was returned by adding 5 ml of

hydrochloric acid (20% vol/vol). Synthetic hard water (sHW) was a mixture of two solutions: (i) $MgCl_2$ and $CaCl_2$ and (ii) $NaHCO_3$ (11). The sHW mixture deviated from the recipe of Helrich (11) because the pH was specified to be 7.6 to 8.0, but pH was a treatment in these experiments.

Each treatment evaluated in the experiment was a combination of up to eight factors: pH, sHW, NH_4, NO_3, Mn, Cu, Zn, and/or Fe. A single replication of all factors at all levels of the full factorial design could not be run in a single day, so results from single-factor trials were used as the basis to combine Cu, Fe, Mn, and Zn as a single factor (divalent metal ion solution) and NH_4, NO_3, and sHW as a single factor (nitrogen and hard water solution) (6). Cu, Fe, Mn, and Zn were combined as a single factor, because Mn was determined to be highly influential on the biocidal activity of ClO_2, Fe to have a small but negligible effect, and Cu and Zn to have no effect on ClO_2 activity when each was tested as a single factor at 5 mg/liter of the metal ion. The concentrations of Cu, Fe, Mn, and Zn in these solutions were equal. Ammonium, NO_3, and sHW were combined as a single factor, because each had no effect on ClO_2 activity when tested as a single factor (6). A nitrogen and hard water solution with 100 mg/liter contained 200 mg N per liter with 100 mg N per liter from $(NH_4)_2SO_4$, 100 mg N per liter from KNO_3 (granular), and 100 mg/liter of $CaCO_3$ equivalent. While effects from individual nutrients were confounded by combining these factors in one solution, this allowed for a complex solution that contained all factors while limiting the number of treatments. Each treatment combination was made as a separate solution and pipetted into culture tubes with the appropriate certified pH buffer solution as the base liquid.

Chlorine dioxide. A ClO_2 stock solution was manufactured daily as needed by mixing IVR-SAN 15 (15% sodium chlorite and 4.5% sodium chloride) and Activator-H (14.4% wt/wt hydrochloric acid) (CH_2O International, Inc., Olympia, WA). The chemical reaction:

$$5NaClO_2 + 4HCl \rightarrow 4ClO_2 + 5NaCl + 2H_2O$$
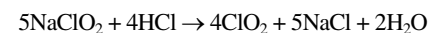
results in the production of ClO_2 with lower chlorate levels than other methods of production (1). An iodometric titration method was used to determine ClO_2 concentration of the stock solution, which ranged from 26,000 to 32,000 mg/liter (9). Chlorine dioxide stock solution was produced in an amber glass bottle, capped with a Parafilm-covered rubber stopper at all times, and stored at 5°C to inhibit photolytic degradation and volatilization. If two replications were to be done in a day, the stock solution was divided equally and stored at 5°C. During each experiment, the ClO_2 stock solution being used was partially submersed in iced water and quickly recapped after withdrawal of each aliquot.

Suspension tests. Test factors and the levels of each test factor evaluated for efficacy against micro- and macroconidia of *F. oxysporum* f. sp. *narcissi* and conidia of *T. basicola* included: ClO_2 at 0, 1, 3, 5, 7, and 9 mg/liter; the divalent metal ion solution at 0, 1, 3, and 5 mg/liter; the nitrogen and hard water solution at 0 and 100 mg/liter; and pH 5 and 8. Test factors and their levels evaluated for efficacy against aleuriospores of *T. basicola* were ClO_2 at 0, 22, 34, 46, 58, and 70 mg/liter; the divalent metal ion solution at 0 and 5 mg/liter; the nitrogen and hard water solution at 0 and 100 mg/liter; and pH 5 and 8. All experiments were repeated once.

Twenty milliliters of each solution was pipetted into sterile, borosilicate, plain end, culture tubes (16 × 100 mm) capped with Sav-It Tube (Fisher Scientific) flexible red closures (16 mm). The required concentrations of ClO_2 (0 to 70 mg/liter) were produced by adding the appropriate volume of stock solution to 20 ml of the treatment solution in a culture tube. The culture tube was placed on a Labquake tube shaker (16.5 × 27.9 cm) for 10 min. Conidial suspension was added with a 1-ml disposable plastic syringe, using a 23-gauge hypodermic needle, by piercing the Sav-It Tube closure on top of the culture tube. The culture tube was placed back on the shaker for 30 s. Contents of the culture tube were poured through a sterile 12.5-cm-diameter P-2 filter paper (Fisher Scientific) pressed into a Coors porcelain Büchner funnel with a fixed perforated plate (100 mm diameter), which was connected to a Welch vacuum pump. When the solution had been drawn completely down through the filter paper, 50 ml of sterile, deionized water was added to flush ClO_2 from the filter. The filter paper was removed and cut with scissors to leave a 5.08 cm^2 piece that was inverted onto the surface of half-strength PDA (½-PDA) in a 15 × 100 mm plastic petri plate. Between treatments, the inner cup of the Büchner funnel was rinsed with a 10% bleach solution (5.25% sodium hypochlorite); counter surfaces were sprayed with 95% ethanol and wiped off; and tweezers and scissors were dipped in 95% ethanol and flamed.

Petri plates were stacked two to four high in rows with each successive layer staggered by half a plate. The following day, the filter paper was removed, and >100 spores were checked for germination within the middle of the area in which the filter was placed. Plates were placed back under fluorescent lights in the same stacking pattern. On the third day after plating, spore germination was evaluated. By day 3, germination was more difficult to evaluate in plates with significant mycelial growth.

Experimental design. A randomized complete block design with four replications, blocked by time, was used in all three experiments. Experiments with conidia of *F. oxysporum* f. sp. *narcissi*, co-

nidia of *T. basicola*, and aleuriospores of *T. basicola* had 48, 33, and 24 treatments, respectively. A replication consisted of one culture tube containing the specific water solution (pH, nitrogen and hard water solution, and divalent metal ion solution) and ClO_2 concentration.

The number of treatment factors and levels were restricted to the number that could be run within a 2.5-h period. The concentration of ClO_2 in the stock solution did not diminish during this period but did diminish within a 4-h period. As a result, variation among treatments was partially controlled by blocking each replication within a 2.5-h period.

Preliminary experiments were done to determine the range in concentrations of ClO_2 that would result in percent ungerminated spores >0 and <100 at multiple ClO_2 concentrations, for calculating a dose curve response. Because of the use of solutions with combined factors and partial treatment selection, a potential existed for unanticipated interactions; therefore all treatment factors and levels were tested at a single ClO_2 level (3 mg ClO_2 per liter) that was likely to result in an intermediate level of biocide activity in each experiment.

Statistical analysis. The SAS probit analysis (PROC PROBIT), a nonlinear regression that accounts for unequal variances, was used to obtain prediction values and confidence limits (CLs) (SAS Institute, Cary, NC). The LD_{50} provides the highest precision for statistical comparisons of the commonly used dose-response design; therefore, LD_{50} values were compared based on confidence limits (25). Insufficient evidence existed to reject the null hypothesis of equal means (H_0: $LD_{50\ 1}$ = $LD_{50\ 2}$) when confidence limits of LD_{50}s overlapped. In addition, the variance of LD_{50}s was compared based on slopes and associated standard errors for each treatment combination, e.g., a significantly larger slope indicates a smaller variance, which means a greater change in biocidal activity of ClO_2 per unit change in concentration of ClO_2 compared with a smaller slope. With a smaller slope, more units of ClO_2 are required to reach a higher LD, such as the LD_{90}, than with a larger slope (25). Slopes were compared based on a *t'* test (20). If the *t'* value, calculated by

$$t' = (\beta_1 - \beta_2) \div [s^2(\beta_1) + s^2(\beta_2)]^{1/2}$$

was less than or equal to the t_α value of

$$t_\alpha = t[0.05; \eta_1 + \eta_2 - (2p)]$$

then insufficient evidence existed to reject the null hypothesis of equal slopes (H_0: β_1 = β_2), where β_1 and β_2 are the slopes being compared, $s^2(\beta_1)$ and $s^2(\beta_2)$ are the standard deviations of β_1 and β_2, respectively, η_1 and η_2 are the total numbers of observations used to calculate β_1 and β_2, respectively, *p* is the number of parameters being compared, and *t* is the critical value from a

t table with a significance level of 0.05 and $\eta_1 + \eta_2 - (2p)$ degrees of freedom (20). LD_{90} values were not used in the statistical analysis but are provided in the results because they are more representative of rates of ClO_2 that would be used to treat irrigation water commercially than the LD_{50} values.

For *F. oxysporum* f. sp. *narcissi*, significance between experiments and all other factors was tested by an analysis of variance procedure using PROC MIXED (SAS Institute). For both spore types of *T. basicola*, significance between experiments was tested by comparison of LD_{50} values, with experiments being not significantly different only if the same factors had overlapping confidence intervals of LD_{50} values.

Interactions between the nitrogen and hard water solution, the divalent metal ion solution, and pH were tested based on a regression of mortality and divalent metal ion solution dose at 3 mg ClO_2 per liter using the divalent metal ion solution concentrations versus the concentration of ClO_2 as the dependent variable. As a result, the slope was negative, but the lethal dose values were used only as a means to compare treatments. This was termed a secondary lethal dose ($2°\ LD_{50}$) because mortality was a measure of the LD_{50} caused by 3 mg ClO_2 per liter after it had reacted with the divalent metal ion solution and was not due directly to the divalent metal ion solution dose.

A low $2°\ LD_{50}$ means the divalent metal ion solution was highly reactive with ClO_2, so only a relatively small concentration of the divalent metal ion solution was required to reduce ClO_2 activity. A high $2°\ LD_{50}$ means the divalent metal ion solution was less reactive with ClO_2, so a relatively large concentration of the divalent metal ion solution was required before it reduced ClO_2 activity.

RESULTS

Activity of ClO_2 against macro- and microconidia of *F. oxysporum* f. sp. *narcissi*. Several factors in experiments 1 and 2 had only values of 0 and 100% mortality with differences in dose of 2 mg/liter but not consistently the same factors; therefore the SAS procedure of Proc Mixed was used initially to test significance of factors. Experiment ($P = 0.5360$) and spore type ($P = 0.8722$) were not significantly different for *F. oxysporum* f. sp. *narcissi*, while all other factors were significant ($P < 0.0001$). Data for both experiments and macro- and microconidia were combined for the probit analysis. LD_{50} values were significantly higher with each incremental increase in concentration of divalent metal ion solution at pH 5 and 8 (Table 1). LD_{50} values at pH 5 were significantly lower than at pH 8 when other factors were equal. At pH 8, an increase from 0 to 100 mg concentration of the nitrogen and hard water solution per

liter resulted in lower LD_{50} values and indicated a negative interaction between the nitrogen and hard water solution and ClO_2 (Table 1). Probit curves could not be calculated for the treatments of pH 5 and 0 mg concentration of the nitrogen and hard water solution per liter, because no intermediate mortality values existed. For these treatments, mortality changed from 0 to 100% with an increase to the next ClO_2 level, i.e., 2 mg ClO_2 per liter. Results were not significantly different between day 1 and day 3 counts for spore germination. Plates with a very high percent spore germination on day 1 were difficult to count on day 3. Because the spore germination counts for day 1 were more accurate than for day 3, only data from day 1 were analyzed by Probit procedures.

Secondary lethal dose values that cause 50% mortality of spores at pH 8 were significantly lower than at pH 5 for both 0 and 100 mg of the nitrogen and hard water solution per liter (Table 2). An increase from 0 to 100 mg of the nitrogen and hard water solution per liter corresponded with a lower $2°$ LD_{50} at pH 5 and a slightly higher $2°$ LD_{50} at pH 8.

Activity of ClO_2 against conidia of *T. basicola*. Experiments 1 and 2 were significantly different. Similar trends in mortality were found with conidia of *T. basicola* as found with conidia of *F. oxysporum* f. sp. *narcissi*. Lethal dose values that cause 50% mortality of spores were significantly higher with each incremental increase in concentration of the divalent metal ion solution at pH 5 and 8 (Table 3). Lethal dose values that cause 50% mortality of spores were significantly lower at pH 5 than at pH 8 when other factors were equal, except at 0 mg/liter of the nitrogen and hard solution and divalent metal ion solution. At pH 8 and pH 5 in experiment 1, an increase from 0 to 100 mg of the nitrogen and hard solution per liter resulted in lower LD_{50} values and indicated a negative interaction between the nitrogen and hard

solution and ClO_2 (Table 3). In the second experiment, LD_{50} values were not available at pH 5 and 0 mg of the nitrogen and hard solution per liter because no intermediate mortality values existed. Mortality changed from 0% at 1 mg ClO_2 per liter to 100% at 3 mg ClO_2 per liter at both 0 and 5 mg of the divalent metal ion solution per liter. Data were not significantly different between day 1 and day 3 spore germination counts.

Trends in LD_{50} values were the same for conidia of *F. oxysporum* f. sp. *narcissi* as they were with conidia of *T. basicola*. Secondary lethal dose values that cause 50% mortality of spores at pH 8 were significantly lower than at pH 5 for both 0 and 100 mg of the nitrogen and hard water solution per liter (Table 4). An increase from 0 to 100 mg of the nitrogen and hard water solution per liter corresponded with a lower $2°$ LD_{50} at pH 5 in both experiments and a slightly higher $2°$ LD_{50} at pH 8 in experiment 1 only (Table 4).

Table 1. Probit prediction of chlorine dioxide (ClO_2) concentration (mg/liter) required to cause 50% mortality (LD_{50}) when ClO_2 had reacted with the specified factors for 10 min before treatment of the macro- and microconidia of *Fusarium oxysporum* f. sp. *narcissi* for 30 s

| | Treatments | | | | | | | |
pH	N-sHW[a] (mg/liter)	Cu-Fe-Mn-Zn[b] (mg/liter)	LD_{50}[c]	95% confidence limit	Slope[d]	Standard error of the slope	n[e]	LD_{90}[f]
8	0	0	1.40	1.28 - 1.51	1.69	0.0665 d	24	2.15
8	0	5	6.96	6.91 - 7.01	1.24	0.0442 f	48	8.00
5	100	0	0.50	0.47 - 0.53	4.54	0.0381 b	36	0.78
5	100	3	1.99	1.93 - 2.05	1.16	0.0312 f	48	3.09
5	100	5	2.98	2.89 - 3.06	0.72	0.0196 g	60	4.75
8	100	0	0.58	0.54 - 0.62	5.06	0.2367 a	36	0.83
8	100	1	1.89	1.79 - 2.00	2.83	0.1606 c	24	2.34
8	100	3	2.54	2.48 - 2.59	1.40	0.0238 e	48	3.45
8	100	5	4.27	4.19 - 4.35	0.62	0.0149 g	60	6.33

[a] Concentration (mg/liter) of each: nitrogen from potassium nitrate, nitrogen from ammonium sulfate, and calcium carbonate equivalent (the unit for quantifying hard water).

[b] Concentration (mg/liter) of each: copper, iron, manganese, and zinc.

[c] Lethal dose of ClO_2 resulting in 50% mortality of spores as predicted by Proc Probit (SAS Institute). Treatments with overlapping confidence limits at the LD_{50} are not significantly different. All probit curves had a χ^2 probability of <0.0001.

[d] Slope parameter and standard error of the slope were generated by probit analysis. Slopes were compared based on a t distribution. Slopes with the same letter are not significantly different ($P = 0.05$).

[e] Number of samples (ClO_2 levels × replications) per calculation.

[f] LD_{90} values are listed as a reference to the ClO_2 level likely to be used commercially to treat irrigation water.

Table 2. Probit prediction of concentration of a divalent metal ion solution required to interact negatively with the biocidal activity of 3 mg ClO_2 per liter so the remaining ClO_2 will cause a 50% mortality (LD_{50}) when the divalent metal ion solution, ClO_2, and specified factors had reacted for 10 min before treatment of macro- and microconidia of *Fusarium oxysporum* f. sp. *narcissi* for 30 s

| | Treatments | | | | | | |
pH	N-sHW[a] (mg/liter)	ClO_2 (mg/liter)	$2°$ LD_{50}[b]	95% confidence limit	Slope[c]	Standard error of the slope	n[d]	Probability > chi-square[e]
5	0	3	12.95	9.12 - 53.14	–0.32	0.1364 a	48	0.0179
5	100	3	5.22	4.76 - 6.00	–0.65	0.1183 ab	48	<0.0001
8	0	3	2.40	1.35 - 2.91	–1.65	0.5543 b	48	0.0030
8	100	3	3.81	3.32 - 4.32	–0.90	0.1782 b	48	<0.0001

[a] Concentration (mg/liter) of each: nitrogen from potassium nitrate, nitrogen from ammonium sulfate, and calcium carbonate equivalent (the unit for quantifying hard water).

[b] A secondary lethal dose value ($2°$ LD) is different from a lethal dose value typically calculated with a probit analysis, because mortality was a measure of 50% mortality caused by 3 mg ClO_2 per liter after it had reacted with the divalent metal ion solution and was not caused by the divalent metal ion solution. The $2°$ LD values were calculated only to compare treatments. Analysis was done using Proc Probit (SAS Institute). Treatments with overlapping confidence limits at the LD_{50} are not significantly different.

[c] Slope parameter and standard error of the slope were generated by probit analysis. Slopes were compared based on a t distribution. Slopes with the same letter are not significantly different ($P = 0.05$).

[d] Number of samples (ClO_2 levels × replications) per calculation.

[e] Chi-square (χ^2) probability associated with a probit curve.

Activity of ClO₂ against aleuriospores of *T. basicola*. Experiments 1 and 2 were significantly different. Similar trends in mortality were found with aleuriospores of *T. basicola* as were found with conidia of *T. basicola* and *F. oxysporum* f. sp. *narcissi*. The notable difference was that considerably higher levels of ClO₂ were required to kill aleuriospores of *T. basicola* than the other fungal propagules tested in these experiments. Because preliminary experiments demonstrated the need for very high rates of ClO₂ and a wide range in rates of ClO₂, treatment selection was restricted even more than for the other propagules by combining the nitrogen and hard water solution (0 and 100 mg/liter) and divalent metal ion solution (0 and 5 mg/liter) treatments. LD₅₀ values were significantly higher for aleuriospores of *T. basicola* with an increase in concentration of the divalent metal ion solution and the nitrogen and hard water solution, except at pH 5 in the second experiment (Table 5). All the LD₅₀ values at pH 5 were significantly lower than at pH 8 when other factors were equal. Data were not signifi-

Table 3. Probit prediction of chlorine dioxide (ClO₂) concentration (mg/liter) required to cause 50% mortality (LD₅₀) when ClO₂ had reacted with the specified factors for 10 min before treatment of conidia of *Thielaviopsis basicola* for 30 s

Experiment	pH	Treatment factors N-sHW[a] (mg/liter)	Cu-Fe-Mn-Zn[b] (mg/liter)	LD₅₀[c]	95% confidence limit	Slope[d]	Standard error of the slope	n[e]	LD₉₀[f]
1	5	0	0	1.29	1.10 - 1.50	1.64	0.1186 b	8	2.07
	5	0	5	1.73	1.57 - 1.19	0.73	0.0329 e	12	3.50
	8	0	0	1.20	1.02 - 1.41	1.58	0.1195 b	8	2.01
	8	0	5	11.80	10.43 - 13.95	0.19	0.0233 c	12	18.40
	5	100	0	0.53	0.47 - 0.57	2.95	0.0381 a	12	0.96
	5	100	1	1.01	0.91 - 1.10	1.30	0.1259 c	8	2.05
	5	100	3	1.89	1.77 - 2.00	0.90	0.0424 d	16	3.32
	5	100	5	3.08	2.90 - 3.25	0.47	0.0187 f	20	5.78
	8	100	0	0.99	0.93 - 1.05	1.89	0.1294 b	12	1.67
	8	100	1	1.99	1.90 - 2.08	1.76	0.0813 b	8	2.72
	8	100	3	3.61	3.51 - 3.72	0.98	0.0449 d	16	4.92
	8	100	5	5.56	5.40 - 5.72	0.47	0.0184 f	20	8.31
2	8	0	0	1.40	1.22 - 1.58	1.61	0.0094 d	8	2.19
	8	0	5	7.73	7.63 - 7.84	1.08	0.0486 e	16	9.10
	5	100	0	0.54	0.49 - 0.58	3.13	0.0394 a	12	0.95
	5	100	3	2.41	2.29 - 2.53	0.82	0.0370 f	16	3.97
	5	100	5	3.58	3.39 - 3.77	0.38	0.0150 h	20	6.91
	8	100	0	1.01	0.95 - 1.08	1.91	0.1344 c	12	1.68
	8	100	1	1.96	1.85 - 2.07	2.34	0.1350 b	8	2.51
	8	100	3	4.63	4.52 - 4.74	0.89	0.0384 f	16	6.08
	8	100	5	5.94	5.81 - 6.07	0.65	0.0248 g	20	7.92

[a] Concentration (mg/liter) of each: nitrogen from potassium nitrate, nitrogen from ammonium sulfate, and calcium carbonate equivalent (the unit for quantifying hard water).
[b] Concentration (mg/liter) of each: copper, iron, manganese, and zinc.
[c] Lethal dose of ClO₂ resulting in 50% mortality of spores as predicted by Proc Probit (SAS Institute). Treatments with overlapping confidence limits at the LD₅₀ are not significantly different. All probit curves had a χ^2 probability of <0.0001.
[d] Slope parameter and standard error of the slope were generated by probit analysis. Slopes were compared based on a *t* distribution. Slopes with the same letter are not significantly different ($P = 0.05$).
[e] Number of samples (ClO₂ levels × replications) per calculation.
[f] LD₉₀ values are listed as a reference to the ClO₂ level likely to be used commercially to treat irrigation water.

Table 4. Probit prediction of the concentration of a divalent metal ion solution required to interact negatively with the biocidal activity of 3 mg ClO₂ per liter so the remaining ClO₂ will cause a 50% mortality (LD₅₀) when the divalent metal ion solution, ClO₂, and specified factors had reacted for 10 min before treatment of conidia of *Thielaviopsis basicola* for 30 s

Experiment	pH	Treatment factors N-sHW[a] (mg/liter)	ClO₂[b] (mg/liter)	2° LD₅₀[c]	95% confidence limit[d]	Slope[e]	Standard error of the slope	n[f]
1	5	0	3	14.52	10.64 - 27.22	−0.20	0.0541 a	16
	5	100	3	5.77	5.51 - 6.11	−0.58	0.0457 b	16
	8	0	3	2.18	2.09 - 2.28	−1.10	0.0429 c	16
	8	100	3	2.49	2.39 - 2.60	−0.99	0.0405 c	16
2	5	0	3	20.25	12.3 - 89.56	−0.14	0.0576 a	16
	5	100	3	5.60	5.31 - 5.96	−0.45	0.0306 b	16
	8	0	3	1.78	1.77 - 1.97	−1.57	0.0695 c	16
	8	100	3	1.94	1.90 - 2.10	−1.93	0.0897 d	16

[a] Concentration in solution (mg/liter) of each: nitrogen from potassium nitrate, nitrogen from ammonium sulfate, and calcium carbonate equivalent (the unit for quantifying hard water).
[b] Concentration in solution (mg/liter) of chlorine dioxide.
[c] A secondary lethal dose value (2° LD) is different from a lethal dose value typically calculated with a probit analysis, because mortality was a measure of 50% mortality caused by 3 mg ClO₂ per liter after it had reacted negatively with the divalent metal ion solution and was not caused by the divalent metal ion solution. The 2° LD values were calculated only to compare treatments.
[d] Treatment differences were determined by overlapping confidence limits at the lethal dose resulting in 50% spore mortality (LD₅₀), a *t* distribution of the slopes of the probit curves, and the corresponding standard error of the slopes. Analysis was done using Proc Probit (SAS Institute).
[e] The slope parameter and standard error of the slope were generated by the probit analysis. Slopes were compared based on a *t* distribution. Slopes with the same letter are not significantly different ($P = 0.05$).
[f] Number of samples (ClO₂ levels × replications) per calculation.

cantly different between day 1 and day 3 spore germination counts.

DISCUSSION

Water properties, such as pH and water hardness, vary across regional and local sources of water, whether from above- or underground sources. In addition, the presence of nutrient leachates in surface-captured water can vary among sites and over time at individual sites. This research demonstrates the need to adjust the rate of ClO_2 according to the demand requirements of the water solution as well as the pathogen and propagule type being targeted. Fungi and types of fungal propagule ranked in order of increasing levels of ClO_2 necessary to achieve mortality were: *F. oxysporum* f. sp. *narcissi* (conidia) ≤ *T. basicola* (conidia) << *T. basicola* (aleuriospores). The factors that affected activity of ClO_2 in the order of decreasing reactivity were concentration of the divalent metal ion solution >> pH > concentration of the nitrogen and hard water solution. However, a 100-mg concentration of the nitrogen and hard water solution per liter interacted with pH to determine the need to increase and decrease ClO_2 dose at pH 5 and 8, respectively, in order to maintain equal biocidal activity.

Chlorine dioxide rates needed to achieve LD_{50} values were similar for conidia of *F. oxysporum* f. sp. *narcissi* and *T. basicola*. Roberts and Reymond (24) noted mortality of *Cryptosporiopsis perennans*, *Mucor piriformis, Penicillium expansum*, and *Botrytis cinerea* spores reached 100.0, 100.0, 99.2, and 93.9%, respectively, from 30 s exposure to 3 mg ClO_2 per liter. Hong (13) observed 100% mortality of zoospores of *Phytophthora nicotianae* from 2 mg free Cl per liter (from Cl gas). It is likely that many fungal spores are killed by similar rates of chlorine in solution. Of concern were the high LD_{50} values for

aleuriospores of *T. basicola*. While it was not the objective in this project to test ClO_2 against all pathogens disseminated in water, this demonstrated the need to clarify rates of ClO_2 needed for control of other thick-walled propagules, such as oospores and chlamydospores.

The largest demand on ClO_2 activity came from the divalent metal ion solutions, which would most commonly occur in water as a micronutrient leachate in catchment ponds in nurseries and recirculated watering systems in greenhouses (2). Significant differences occurred between LD_{50} values of 0 and 1 mg concentration of the divalent metal ion solution per liter, with that narrow range in concentration being representative of what could exist in recaptured water. The reactivity of ClO_2 to divalent cations has been reported (7). In fact, ClO_2 is used to reduce Fe and Mn levels in industrial water use (12).

Results from the use of $2°$ LD_{50} values concur with results from LD_{50} values but provide a broader characterization of the responses, which were consistent for conidia of *T. basicola* and *F. oxysporum* f. sp. *narcissi*. Use of $2°$ LD_{50} values show that the divalent metal ion solution placed a higher demand on ClO_2 at pH 8 than at pH 5, and the nitrogen and hard water solution in the presence of the divalent metal ion solution placed a higher demand on ClO_2 at pH 5 but lowered demand on ClO_2 at pH 8. Interactions between the nitrogen and hard water solution, the divalent metal ion solution, and pH likely involve several different chemical reactions. The chemical nature of these interactions is unclear from this research. It is unknown whether the responses from the nitrogen and hard water solution were due to N, sHW, or the combination. Since nitrogen has been proven by others not to place a demand on ClO_2, sHW or the combination of N with sHW

is more likely to be the important factor(s) in these interactions (8).

In nature, water hardness typically occurs at pH levels between 7 and 8 and is not associated with an acidic pH. Regardless, sHW was tested at pH 5 in this research. Differences in ClO_2 activity due to the nitrogen and hard water solution did occur at pH 5. This reaction was not investigated further but shows that Ca and Mg can place a demand on ClO_2 at an acidic pH. The effect of the nitrogen and hard water solution on ClO_2 activity at 5-mg concentration of the divalent metal ion solution per liter was considerably lower in magnitude (difference of 1.35 mg ClO_2 per liter) than that associated with just the divalent metal ion solution. Although the effects of N and sHW were confounded, the use of the nitrogen and hard water solution as a factor in these trials still provided rate predictions that included compensation for the presence of Ca and Mg in leachates at pH 5.

Length of exposure to disinfestants and temperature, organic matter, soluble organic compounds, and turbidity of the water are other factors reported to influence efficacy of disinfectants, but they were not tested in these experiments (10,12,19). Guidelines for commercial disinfestation of irrigation water could include rate adjustments due to shorter and longer exposure times, which would depend on flow rate and size of an irrigation system. The contribution of organic matter on the total demand load and the effect of temperature on volatility losses are factors that have been important in calculating rates of disinfectants (10). In human waste treatment systems, effluents placed a demand on ClO_2 (7). However, suspended solids had little direct influence on ClO_2, except when ClO_2 could not reach microorganisms that colonized or became embedded in suspended solids. At low tem-

Table 5. Probit prediction of chlorine dioxide (ClO_2) concentration (mg/liter) required to cause 50% mortality (LD_{50}) when ClO_2 had reacted with the specified factors for 10 min before treatment of aleuriospores of *Thielaviopsis basicola* for 30 s

| | | Treatment factors | | | | | | | |
Experiment	pH	Nitrogen[a] (mg/liter)	sHW[b] (mg/liter)	Cu-Fe-Mn-Zn[c] (mg/liter)	LD_{50}[d]	95% confidence limit	Slope[e]	Standard error of the slope	n[f]	LD_{90}[g]
1	5	0	0	0	17.68	12.05 - 21.93	0.04	0.0041 a	24	47.63
	5	100	150	5	33.04	30.05 - 34.28	0.08	0.0045 b	24	48.82
	8	0	0	0	41.71	41.57 - 43.79	0.10	0.0051 c	24	55.59
	8	100	150	5	45.52	44.18 - 46.79	0.08	0.0040 b	24	61.55
2	5	0	0	0	16.09	13.01 - 18.78	0.04	0.0025 a	24	46.90
	5	100	150	5	15.02	12.39 - 17.37	0.04	0.0020 a	24	47.08
	8	0	0	0	25.14	22.66 - 27.39	0.04	0.0020 a	24	57.98
	8	100	150	5	32.67	30.58 - 34.51	0.06	0.0035 b	24	55.30

[a] Concentration in solution (mg/liter) of nitrogen from potassium nitrate and from ammonium sulfate.

[b] Concentration in solution (mg/liter) of calcium carbonate equivalent (the unit for quantifying hard water).

[c] Concentration in solution (mg/liter) of each: copper, iron, manganese, and zinc.

[d] Lethal dose of ClO_2 resulting in 50% mortality of spores as predicted by Proc Probit (SAS Institute). Treatments with overlapping confidence limits at the LD_{50} are not significantly different. The intercept was set at 0.35 for all probit curves except where pH = 5 and sHW = 0, when it was set at 0.7 in experiment 1 and 0.5 in experiment 2. Analysis was done using Proc Probit (SAS Institute). All probit curves had a χ^2 probability of <0.0001.

[e] The slope parameter and standard error of the slope were generated by the probit analysis. Slopes were compared based on a *t* distribution. Slopes with the same letter are not significantly different ($P = 0.05$).

[f] Number of samples (ClO_2 levels × replications) per calculation.

[g] LD_{90} values are listed as a reference to the ClO_2 level likely to be used commercially to treat irrigation water.

peratures, disinfection kinetics are slower. At higher temperatures, ClO_2 is less stable and decays more rapidly. With *Giardia*, 26 and 11 mg ClO_2 per min per liter at 5 and 25°C, respectively, were required to reduce the bacteria population below a 2.5 log inactivation level (12).

Because disinfectants react quickly with ions in solution and decay due to environmental and physical factors, rates need to be high enough to compensate for the load demand so that remaining levels are sufficient to kill different fungal, bacterial, and viral genera and species. In contrast, higher doses increase costs, so knowing the lowest effective rate will help lower costs. The results from this research demonstrate, using a select number of factors, that rate adjustments are important with ClO_2, and some types of fungal propagules are more tolerant of ClO_2 than other pathogens. Additional research would further define these rates of ClO_2 needed to manage plant pathogens in irrigation water. Rates of all disinfectants need to be selected by demand load considerations and the organisms being targeted (3).

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